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ORIGINAL ARTICLE

GZ-793A, a lobelane analog, interacts with the vesicular monoamine transporter-2 to inhibit the effect of methamphetamine

David B. Horton, Justin R. Nickell, Guangrong Zheng, Peter A. Crooks and Linda P. Dwoskin

Department of Pharmaceutical Sciences, College of Pharmacy, University of Kentucky, Lexington, Kentucky, USA

Abstract

(*R*)-3-[2,6-*cis*-Di(4-methoxyphenethyl)piperidin-1-yl]propane-1,2-diol (GZ-793A) inhibits methamphetamine-evoked dopamine release from striatal slices and methamphetamine self-administration in rats. GZ-793A potently and selectively inhibits dopamine uptake at the vesicular monoamine transporter-2 (VMAT2). This study determined GZ-793A's ability to evoke [^{3}H]dopamine release and inhibit methamphetamine-evoked [^{3}H]dopamine release from isolated striatal synaptic vesicles. Results show GZ-793A concentration-dependent [^{3}H]dopamine release; nonlinear regression revealed a two-site model of interaction with VMAT2 (High- and Low-EC_{50} = 15.5 nM and 29.3 μM, respectively). Tetrabenazine and reserpine completely inhibited GZ-793A-evoked [^{3}H]dopamine release, however, only at the High-affinity site. Low concentrations of GZ-793A that interact with the extravesicular dopamine uptake site and the High-affinity intravesicular DA release site also inhibited methamphetamine-evoked [^{3}H]dopamine release from synaptic vesicles. A rightward shift in the methamphetamine concentration-response was evident with increasing concentrations of GZ-793A, and the Schild regression slope was 0.49 ± 0.08, consistent with surmountable allosteric inhibition. These results support a hypothetical model of GZ-793A interaction at more than one site on the VMAT2 protein, which explains its potent inhibition of dopamine uptake, dopamine release via a High-affinity tetrabenazine- and reserpine-sensitive site, dopamine release via a Low-affinity tetrabenazine- and reserpine-insensitive site, and a low-affinity interaction with the dihydrotetrabenazine binding site on VMAT2. GZ-793A inhibition of the effects of methamphetamine supports its potential as a therapeutic agent for the treatment of methamphetamine abuse.

Keywords: dopamine release, dopamine uptake, methamphetamine, neuropharmacology, pharmacotherapy, VMAT2.
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Methamphetamine (METH) abuse is a serious public health concern. According to the 2010 National Survey on Drug Use and Health, over 350 000 people in the United States reported using METH in the past month (Substance Abuse and Mental Health Services Administration 2011). Currently, no FDA-approved pharmacotherapies are available to treat METH abuse. METH produces reward by increasing extracellular dopamine (DA) concentrations through DA transporter (DAT)-mediated reverse transport (Fischer and Cho 1979; Wise and Bozarth 1987; Di Chiara and Imperato 1988). METH increases cytosolic DA available for DAT-mediated reverse transport though an interaction with the vesicular monoamine transporter-2 (VMAT2), inhibiting DA uptake at VMAT2 and promoting DA release from synaptic vesicles, as well as by inhibiting monoamine oxidase (Sulzer and Rayport 1990; Pifl *et al.* 1995; Sulzer *et al.* 2005). Under normal conditions, VMAT2 functions to take up DA from the cytosol into synaptic vesicles, thereby maintaining a dynamic equilibrium to counteract the passive leakage of DA from the vesicles (Eisenhofer *et al.* 2004). METH has been suggested to be transported into synaptic vesicles through VMAT2 and passive diffusion across the vesicular

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Address correspondence and reprint requests to Linda P. Dwoskin, College of Pharmacy, University of Kentucky, Lexington, KY 40536-0082, USA. E-mail: ldwoskin@email.uky.edu

Abbreviations used: DA, dopamine; DAT, dopamine transporter; DTBZ, dihydrotetrabenazine; GZ-793A, (*R*)-3-[2,6-*cis*-di(4-methoxyphenethyl)piperidin-1-yl]propane-1,2-diol; METH, methamphetamine; PEI, polyethyleneimine; TBZ, tetrabenazine; VMAT2, vesicular monoamine transporter-2.

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membrane, and to release DA from synaptic vesicles through a facilitated diffusion mechanism that is characteristic of carrier proteins (Fischer and Cho 1979; Sulzer *et al.* 2005). Thus, uptake of METH (as a substrate) by the transporter increases the occurrence of DA binding to sites on the internal face of the transporter. As the protein undergoes a conformational change, DA is translocated to the cytosolic face of the protein, and bound DA is released into the cytosol. As monoamine oxidase activity is inhibited by METH, the increased cytosolic DA is available for reverse transport by DAT leading to increased extracellular DA concentrations.

In addition, METH, a weak base, disrupts the pH gradient of synaptic vesicles, which also leads to DA release from vesicles into the cytosol (Sulzer and Rayport 1990; Schuldiner *et al.*, 1993; Sulzer *et al.* 2005). However, the weak base explanation for METH-evoked DA release from vesicles has been challenged by observations that weak bases, including chloroquine and bafilomycin, decrease the pH gradient twofold more than METH, while releasing DA from vesicles 50% less than METH (Sulzer *et al.* 2005). Moreover, the S(+)-isomer of amphetamine is more effective at releasing DA compared with the R(−)-isomer, despite that these enantiomers have an identical weak base effect (Peter *et al.* 1995; Sulzer *et al.* 2005). Regardless of the specific mechanism, one of the primary sites of METH action is VMAT2, which increases cytosolic DA available for reverse transport at DAT, thus leading to increased extracellular DA concentrations and reward.

Based on the role of VMAT2 in METH effects, drug discovery efforts have focused on VMAT2 as a pharmacological target for the development of novel compounds to treat METH abuse. Lobeline (Fig. 1), the principal alkaloid of *Lobelia inflata*, inhibits [^{3}H]dihydrotetrabenazine (DTBZ) binding to VMAT2, [^{3}H]DA uptake at VMAT2, and METH-evoked DA release from striatal slices (Teng *et al.* 1997, 1998; Nickell *et al.* 2010). Lobeline inhibits METH-induced hyperactivity, behavioral sensitization and METH self-administration in rats, supporting its potential as a treatment for METH abuse (Harrod *et al.* 2001, 2003; Dwoskin and Crooks 2002). Lobeline has been evaluated in clinical trials for this indication and found to be safe in METH addicts (Jones 2007). Importantly, lobeline has limited selectivity for VMAT2, exhibiting high affinity for nicotinic acetylcholine receptors (Damaj *et al.* 1997; Miller *et al.* 2004).

Structure-activity relationships revealed that lobelane (Fig. 1), a saturated, chemically defunctionalized lobeline analog, exhibited low affinity for nicotinic receptors and enhanced affinity and selectivity for VMAT2 compared to its parent compound (Miller *et al.* 2004; Nickell *et al.* 2010). Lobelane also inhibited METH-evoked DA release from striatal slices and decreased METH self-administration in rats (Neugebauer *et al.* 2007; Nickell *et al.* 2010). However, tolerance developed to the behavioral effects of lobelane (Neugebauer *et al.* 2007). Furthermore, the physicochemical properties of lobelane were not optimal. Low water solubility limited the development of lobelane with respect to clinical investigation.

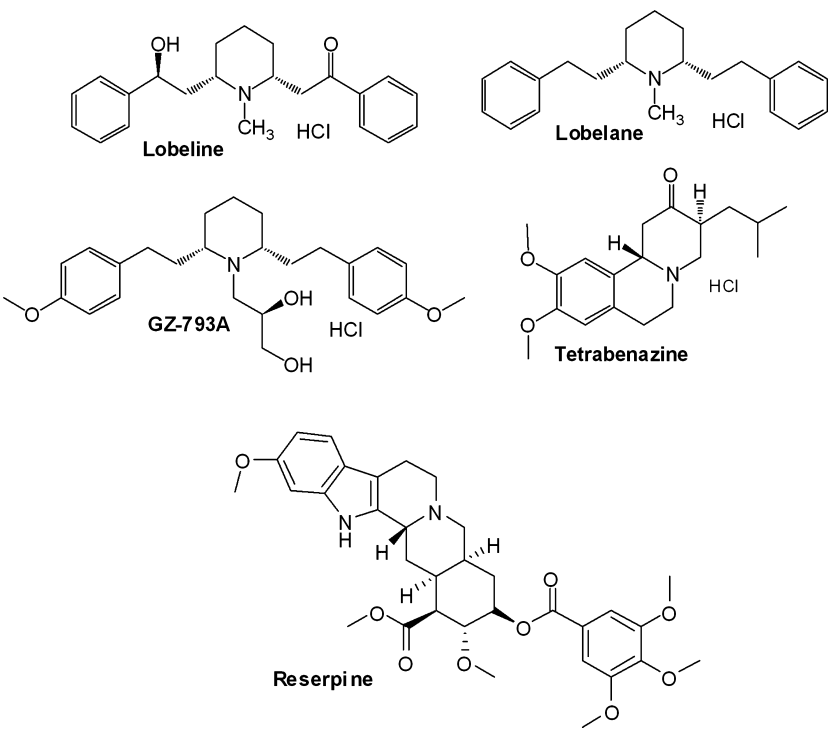

Fig. 1 Chemical structures of lobeline, lobelane, GZ-793A, tetrabenazine (TBZ), and reserpine. Lobeline is the principal alkaloid found in *lobelia inflata*. Lobelane is the chemically defunctionalized, saturated analog of lobeline. GZ-793A is a *para*-methoxy analog of lobelane incorporating an *N*-propan-1,2-diol moiety. TBZ is a benzoquinolizine compound and vesicular monoamine transporter 2 (VMAT2) inhibitor proposed to interact with a site distinct from the dopamine (DA) uptake site on VMAT2. Reserpine is an indole alkaloid and VMAT2 inhibitor, proposed to interact with the DA uptake site on VMAT2.

Structural modification of lobelane was pursued with the aim of improving water solubility. Replacement of the *N*-methyl group of lobelane with a *N*-propan-1,2-diol moiety afforded the lead analog, (*R*)-3-[2,6-*cis*-di(4-methoxyphenethyl)piperidin-1-yl]propane-1,2-diol (GZ-793A) (Fig. 1). GZ-793A potently and selectively inhibited DA uptake at VMAT2 via a competitive mechanism of action (Horton *et al.* 2011). Furthermore, GZ-793A decreased METH-evoked DA release from striatal slices, without altering field stimulation- and nicotine-evoked DA release, indicating specific inhibition of the effects of METH (Horton *et al.* 2011; unpublished observations). Importantly, GZ-793A specifically decreased METH self-administration without altering food-maintained responding (Beckmann *et al.* 2012). Thus, the ability of GZ-793A to inhibit METH *in vitro* translated into efficacy inhibiting METH in the *in vivo* animal model. However, the cellular mechanism underlying the GZ-793A-induced inhibition of METH both *in vitro* and *in vivo* has not been evaluated fully. This study determined the ability of GZ-793A to inhibit the effects of METH to release DA from isolated synaptic vesicles. Considering that VMAT2 is a primary target for the mechanism of action of METH, the ability of GZ-793A to evoke [^{3}H]DA release and inhibit METH-evoked [^{3}H]DA release from vesicles was investigated, and these effects were compared to those of the classical VMAT2 inhibitors, TBZ and reserpine.

Materials and methods

Animals

Male Sprague–Dawley rats (200–250 g; Harlan, Indianapolis, IN, USA) were housed two per cage with *ad libitum* access to food and water in the Division of Laboratory Animal Resources at the University of Kentucky (Lexington, KY, USA). Experimental protocols involving the animals were in accord with the 1996 *NIH Guide for the Care and Use of Laboratory Animals* and were approved by the Institutional Animal Care and Use Committee at the University of Kentucky. This study follows the ARRIVE guidelines set forth to ensure accurate reporting of the experiments conducted in this study.

Materials

[^{3}H]DA (dihydroxyphenylethylamine, 3,4-[7-^{3}H]; specific activity, 28 Ci/mmol) was purchased from PerkinElmer, Inc. (Boston, MA, USA). ATP-Mg^{2+}, DA, EDTA, EGTA, HEPES, $MgSO_4$, polyethyleneimine (PEI), KOH, potassium tartrate, reserpine, METH and sucrose were purchased from Sigma-Aldrich, Inc. (St. Louis, MO, USA). Ascorbic acid and $NaHCO_3$ were purchased from Aldrich Chemical Co. (Milwaukee, WI, USA). Complete counting cocktail 3a70B was purchased from Research Products International Corp. (Mount Prospect, IL, USA). TBZ was a generous gift from Hoffman-LaRoche Inc. (Nutley, NJ, USA). All compounds were dissolved initially in MilliQ water (GZ-793A and METH at 10 mM; TBZ and reserpine at 1 mM), and then diluted in assay buffer to achieve final concentrations.

Vesicular [^{3}H]DA release assay

GZ-793A- and METH-evoked vesicular [^{3}H]DA release were determined using previously described methods (Nickell *et al.* 2011). Briefly, for each experiment, fresh rat striata were homogenized in 14 mL of ice-cold 0.32 M sucrose solution containing 5 mM $NaHCO_3$ (pH 7.4) with 10 up-and-down strokes of a Teflon pestle homogenizer (clearance, ~ 0.008). Homogenates were centrifuged at 2000 *g* for 10 min at 4°C and resulting supernatants centrifuged at 10 000 *g* for 30 min at 4°C. Pellets were resuspended in 2.0 mL of 0.32 M sucrose and were transferred to tubes containing 7 mL of milliQ water and homogenized with 5 up-and-down strokes of the Teflon pestle homogenizer. Homogenates were transferred to tubes containing 900 μL of 0.25 M HEPES and 900 μL of 1.0 M potassium tartrate solution and centrifuged at 20 000 *g* for 20 min at 4°C. Resulting supernatants were centrifuged at 55 000 *g* for 60 min at 4°C. Subsequently, 100 μL of 1 mM $MgSO_4$, 100 μL of 0.25 M HEPES and 100 μL of 1.0 M potassium tartrate were added to the supernatant and centrifuged at 100 000 *g* for 45 min at 4°C. Pellets were resuspended in 2.7 mL of assay buffer, containing: 25 mM HEPES, 100 mM potassium tartrate, 50 μM EGTA, 100 μM EDTA, and 1.7 mM ascorbic acid, 2 mM ATP-Mg^{2+} (pH 7.4). Then, [^{3}H]DA (300 μL of 0.3 μM) was added and samples incubated for 8 min at 37°C. The final concentration of [^{3}H]DA (0.03 μM) was selected based on the K_m of DA for VMAT2 and on the methods used in our published reports employing the vesicular [^{3}H]DA release assay (Teng *et al.* 1998; Nickell *et al.* 2011). In the latter studies, spontaneous [^{3}H]DA efflux from synaptic vesicles was stable following 8 min of incubation (Teng *et al.* 1998). Following incubation, samples were centrifuged at 100 000 *g* for 45 min at 4°C and resulting pellets were resuspended in a final volume of 4.2 mL of assay buffer. [^{3}H]DA-preloaded vesicles (180 μL) were added to duplicate tubes in the absence or presence of various concentrations (1 nM – 1 mM; 20 μL) of GZ-793A, METH or reserpine, for a final volume of 200 μL and incubated for 8 min at 37°C. Reactions were terminated by the addition of 2.5 mL of ice-cold assay buffer and rapid filtration through Whatman GF/B filters. Samples were washed three times with assay buffer containing 2 mM $MgSO_4$ in the absence of ATP. Radioactivity retained by the filters was determined by liquid scintillation spectrometry (B1600 TR scintillation counter; Perkin-Elmer, Inc.). GZ-793A-, METH- or reserpine-evoked [^{3}H]DA release was calculated for each concentration of test compound by subtracting the radioactivity remaining on the filter in the presence of compound from the amount of radioactivity remaining on the filter in the absence of compound (control samples) and then dividing by the value for control to determine the % above control.

To determine if GZ-793A-induced [^{3}H]DA release from striatal synaptic vesicles was inhibited by TBZ (TBZ-sensitive) or reserpine (reserpine-sensitive), [^{3}H]DA-preloaded synaptic vesicles (180 μL) were added to duplicate tubes containing a range of concentrations (1 nM – 1 mM) of GZ-793A in the absence and presence of TBZ (35 nM) or reserpine (50 nM), and incubated (final volume, 200 μL) for 8 min at 37°C. Samples were processed as previously described.

To determine if METH-induced [^{3}H]DA release from striatal synaptic vesicles was TBZ- or GZ-793A-sensitive, [^{3}H]DA-preloaded synaptic vesicles (180 μL) were added to duplicate tubes containing a range of concentrations (1 nM – 1 mM) of METH in the absence and

presence of TBZ (30 nM – 10 μM) or GZ-793A (7 nM – 1 μM), and incubated (final volume, 200 μL) for 8 min at 37°C. Samples were processed as previously described.

To determine if GZ-793A-induced inhibition of METH-evoked DA release was the result of a rate-dependent slow-offset dissociation, [^{3}H]DA-preloaded synaptic vesicles (180 μL) were added to duplicate tubes containing a range of concentrations (1 μM – 1 mM) of METH in the absence and presence of GZ-793A (1 μM), and incubated (final volume, 200 μL) for either 8 min or 15 min at 37°C. Samples were processed as previously described.

Data analysis

EC_{50} values for GZ-793A, METH, and reserpine were determined from the concentration-effect curves via an iterative curve-fitting program (Prism 5.0; GraphPad Software Inc., San Diego, CA, USA). EC_{50} values for GZ-793A-evoked [^{3}H]DA release in the presence of TBZ or reserpine were determined also using the Prism 5.0 curve-fitting program. TBZ-induced and GZ-793A-induced inhibition of METH-evoked [^{3}H]DA release were analyzed using separate two-way repeated-measures ANOVA. If significant TBZ × METH or GZ-793A × METH interactions were found, one-way ANOVAs followed by Dunnett's *post hoc* test were performed at each METH concentration to determine the concentrations that decreased METH-evoked [^{3}H]DA release. To determine if the various concentrations of TBZ or GZ-793A increased the log EC_{50} value or decreased the E_{max} for METH compared to the values for these parameters in the absence of inhibitor (control), one-way ANOVAs were conducted followed by Dunnett's *post hoc* test. Schild analyses were performed using the dose ratios (DR) obtained by dividing the EC_{50} for METH-evoked [^{3}H]DA release in the presence of inhibitor by that in the absence of inhibitor. Log (DR-1) was plotted as a function of log inhibitor concentration to provide the Schild regression. The data were fit by linear regression; the slope was determined; linearity was assessed using Prism 5.0. Significant difference from unity was revealed if the 95% confidence intervals (CI) of the slope did not include unity (Kenakin 2006).

To determine if the effect of GZ-793A to inhibit METH was rate dependent, a three-way repeated-measures ANOVA was performed. If significant interactions were found, follow-up ANOVAs were performed to identify the source of the interaction. Differences between EC_{50} values and between E_{max} values were determined using repeated-measures two-way ANOVAs. For all analyses, significance was defined as $p < 0.05$.

Results

Striatal synaptic vesicle [^{3}H]DA capacity under control conditions

Vesicle [^{3}H]DA capacity (in pmol/mg) was determined after the 8-min period of [^{3}H]DA loading and after the final centrifugation step (100 000 *g* for 45 min) just prior to incubation with GZ-793A, METH or reserpine and determination of evoked [^{3}H]DA release. After the 8 min loading step, radioactivity counts were 15 210 ± 2450 DPM and protein levels were 20 ± 2 ng. Following the final centrifugation step, counts were 1470 ± 426 DPM and protein levels were 4 ± 1 ng. Importantly, vesicle content was 69.2 ± 12.5 pmol/mg after the 8 min loading step and 45.7 ± 14.3 pmol/mg after the final centrifugation step. Vesicle content was not significantly different before and after centrifugation. Thus, while considerable loss of vesicles occurred during centrifugation, as evidenced by reductions in counts and protein, synaptic vesicles remaining following centrifugation exhibited capacity similar to that prior to centrifugation. Of note, a sufficient number of counts were retained following centrifugation to allow evaluation of effects on vesicular DA release.

GZ-793A elicits [^{3}H]DA release from striatal synaptic vesicles

GZ-793A-evoked [^{3}H]DA release from isolated striatal synaptic vesicles is illustrated in Fig. 2. Nonlinear regression of the GZ-793A concentration-response revealed a two-site model of GZ-793A interaction with VMAT2 ($R^2 = 0.89$, $p < 0.001$; High and Low EC_{50} = 14.3 ± 4.46 nM and 33.0 ± 4.00 μM, respectively; High and Low E_{max} = 37.5 ± 4.32% and 86.1 ± 2.69% vesicular [^{3}H]DA content, respectively). To evaluate inhibition of the effect of GZ-793A on DA release, the highest concentration (35 nM) of TBZ that did not evoke DA release from isolated vesicles was chosen (Nickell *et al.* 2011). TBZ inhibited only the effect of GZ-793A to release DA via High affinity sites on VMAT2 (Fig. 2). Nonlinear regression revealed a one-site model of GZ-793A interaction with VMAT2 in the presence of TBZ ($R^2 = 0.95$, $p < 0.001$; EC_{50} = 23.7 ± 6.53 μM).

Reserpine exhibited low efficacy releasing [^{3}H]DA from the vesicles (E_{max} = 28.4 ± 7.48%, EC_{50} = 1.44 ± 0.47 μM;

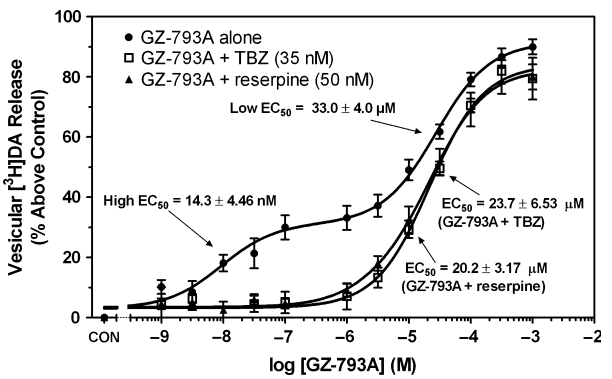

Fig. 2 GZ-793A-evoked [^{3}H]DA release from striatal vesicles fits a two-site model; dopamine (DA) release mediated by the high affinity site is tetrabenazine (TBZ)- and reserpine-sensitive. Data represent the ability of GZ-793A to evoke [^{3}H]DA release from striatal vesicles in the absence (closed circles) and presence of TBZ (35 nM; open squares) or reserpine (50 nM, closed triangle). Control (CON) represents [^{3}H]DA release (2885 ± 532 dpm) in duplicate vesicle aliquots not exposed to GZ-793A. Data are mean (± SEM) [^{3}H]DA release as a percentage of the control. n = 4–8 rats/experiment.

data not shown). The highest concentration (50 nM) of reserpine that did not evoke DA release from isolated vesicles was chosen to evaluate if GZ-793A-evoked [^{3}H]DA release was reserpine-sensitive. Concentration-response curves for GZ-793A-evoked [^{3}H]DA release from striatal vesicles in the absence and presence of reserpine (50 nM) are illustrated in Fig. 2. Reserpine inhibited only the effect of GZ-793A to release DA via High affinity sites on VMAT2. Non-linear regression revealed a one-site model of GZ-793A interaction with VMAT2 in the presence of reserpine ($R^2 = 0.92$, $p < 0.001$; $EC_{50} = 20.2 \pm 3.17$ μM).

TBZ inhibits METH-evoked [^{3}H]DA release from striatal vesicles

The concentration response for METH to evoke [^{3}H]DA release from synaptic vesicles was analyzed using non-linear regression and a significant fit to a single site model was found ($R^2 = 0.95$, $p < 0.001$; Fig. 3). The EC_{50} value for METH was 8.93 ± 1.36 μM and E_{max} was 87.4 ± 1.37%, consistent with our previous findings (Nickell *et al.* 2011). Based on our previous concentration response (Nickell *et al.* 2011), a full range of TBZ concentrations (30 nM – 10 μM) were chosen to evaluate the ability of TBZ to decrease METH-evoked [^{3}H]DA release from synaptic vesicles. TBZ produced a rightward shift in the METH concentration-response curve without a significant reduction in maximal [^{3}H]DA release, consistent with surmountable inhibition. A linear fit ($r^2 = 0.79$, $p < 0.001$) to the Schild regression revealed a slope ($s = 0.92 \pm 0.33$) not significantly different from unity, consistent with competitive inhibition (Fig. 3, inset). Two-way repeated measures ANOVA revealed a main effect of METH [$F_{(11,209)} = 435$, $p < 0.0001$] and TBZ [$F_{(4,19)} = 7.61$, $p < 0.001$], and a METH × TBZ interaction [$F_{(44,209)} = 12.8$, $p < 0.0001$]. To further evaluate the interaction, one-way ANOVAs were conducted at each METH concentration to determine the TBZ concentration which decreased release [METH 3 μM, $F_{(4,20)} = 3.96$, $p < 0.05$; 10 μM, $F_{(4,20)} = 18.2$, $p < 0.0001$; 30 μM, $F_{(4,20)} = 31.1$, $p < 0.0001$; 100 μM, $F_{(4,20)} = 46.4$, $p < 0.0001$; 300 μM, $F_{(4,19)} = 29.3$, $p < 0.0001$; 1 mM, $F_{(4,30)} = 9.39$, $p < 0.001$]. *Post hoc* analyses revealed that at 3 μM METH, only 10 μM TBZ significantly decreased METH-evoked [^{3}H]DA release. At 10 μM–1 mM METH, TBZ (100 nM, 1 μM, and 10 μM) significantly decreased METH-evoked [^{3}H]DA release. Analysis of the log EC_{50} for METH-evoked [^{3}H]DA release revealed that TBZ (100 nM – 10 μM) increased the METH EC_{50} value [Table 1; $F_{(4,20)} = 43.6$, $p < 0.0001$].

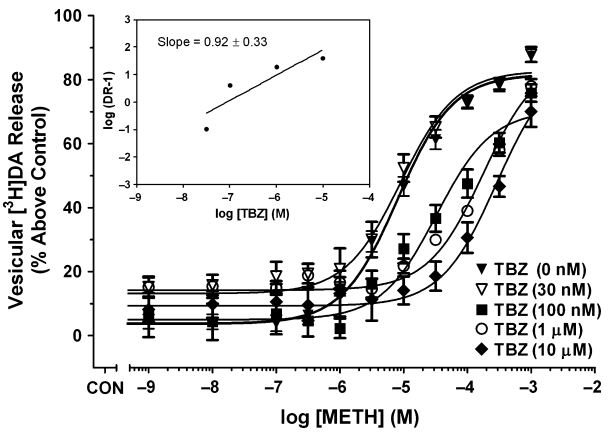

Fig. 3 Tetrabenazine (TBZ) inhibits methamphetamine (METH)-evoked [^{3}H]DA release from striatal vesicles. Data represent the ability of TBZ to inhibit METH-evoked [^{3}H]DA release from striatal vesicles. Control (CON) represents [^{3}H]DA release (3320 ± 335 dpm) in duplicate vesicle aliquots not exposed to METH and TBZ. Data are mean (± SEM) [^{3}H]DA release as a percentage of the control. $n = 4$–9 rats/experiment. Inset shows the Schild regression; log of DR−1 is plotted as a function of log of TBZ concentration.

GZ-793A inhibits METH-evoked [^{3}H]DA release from striatal synaptic vesicles

The concentration-response curve for METH to evoke [^{3}H]DA release from synaptic vesicles is illustrated in Fig. 4. Using non-linear regression, a significant fit to a one-site model was obtained for the METH concentration response ($R^2 = 0.90$, $p < 0.001$). The EC_{50} value for METH was 19.5 ± 5.19 μM and E_{max} was 88.0 ± 1.21%, in agreement with our previous findings (Nickell *et al.* 2011). Figure 4 also illustrates that concentrations of GZ-793A (7 nM – 1 μM) which selectively interact with High-affinity sites on VMAT2 (Fig. 2) inhibited the METH-evoked [^{3}H]DA release. A rightward shift in the METH concentration-response curve was evident with increasing concentrations of GZ-793A, without a significant reduction in maximal [^{3}H]DA release, consistent with surmountable inhibition. A linear fit ($r^2 = 0.95$, $p < 0.001$) to the Schild regression

Table 1 Summary of EC_{50} and E_{max} for METH-evoked [^{3}H]DA release in the absence and presence of TBZ or GZ-793A

	EC_{50} (μM)	E_{max} (%)
TBZ on METH-evoked [^{3}H]DA release		
TBZ (0 nM)	8.93 ± 1.36	82.1 ± 1.21
TBZ (30 nM)	9.89 ± 2.44	83.4 ± 1.23
TBZ (100 nM)	45.5 ± 12.7*	72.0 ± 1.39
TBZ (1 μM)	185 ± 31.1*	88.1 ± 4.55
TBZ (10 μM)	366 ± 115*	91.9 ± 10.3
GZ-793A on METH-evoked [^{3}H]DA release		
GZ-793A (0 nM)	18.9 ± 5.21	85.7 ± 1.22
GZ-793A (7 nM)	11.6 ± 1.37	86.2 ± 0.90
GZ-793A (70 nM)	56.3 ± 6.16*	80.7 ± 3.50
GZ-793A (100 nM)	62.4 ± 8.10*	82.7 ± 2.33
GZ-793A (1 μM)	119 ± 12.2*	79.1 ± 4.02

*$p < 0.05$ different from [^{3}H]DA release in the presence of METH alone and absence of TBZ or GZ-793A.

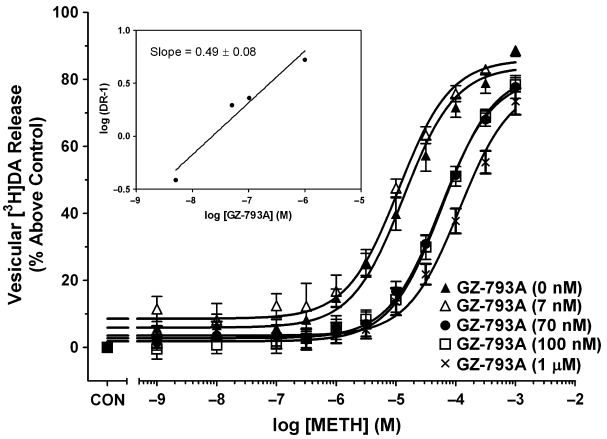

Fig. 4 GZ-793A inhibits methamphetamine (METH)-evoked [^{3}H]DA release from striatal vesicles. Data represent the ability of GZ-793A to inhibit METH-evoked [^{3}H]DA release from striatal vesicles. Control (CON) represents [^{3}H]DA release (2426 ± 277 dpm) in duplicate vesicle aliquots not exposed to METH and GZ-793A. Data are mean (± SEM) [^{3}H]DA release as a percentage of the control. n = 4–10 rats/experiment. Inset shows the Schild regression; log of DR−1 is plotted as a function of log of GZ-793A concentration.

revealed a slope (s = 0.49 ± 0.08) significantly different from unity based on the 95% confidence interval (CI: 0.15 to 0.83), consistent with allosteric inhibition (Fig. 4, inset). Analysis of the concentration response by two-way repeated measures ANOVA revealed main effects of METH [$F_{(11,308)}$ = 821, $p < 0.001$] and GZ-793A [$F_{(4,28)}$ = 8.82, $p < 0.001$], and a METH × GZ-793A interaction [$F_{(44,308)}$ = 8.13, $p < 0.001$]. To further evaluate the interaction, one-way ANOVAS were conducted at each METH concentration to determine the GZ-793A concentrations which decreased release [METH 1 μM, $F_{(4,30)}$ = 3.31, $p < 0.05$; 3 μM, $F_{(4,30)}$ = 9.10, $p < 0.0001$; 10 μM, $F_{(4,30)}$ = 12.9, $p < 0.0001$; 30 μM, $F_{(4,30)}$ = 20.4, $p < 0.0001$; 100 μM, $F_{(4,30)}$ = 25.1, $p < 0.0001$; 300 μM, $F_{(4,28)}$ = 15.6, $p < 0.0001$; 1 mM, $F_{(4,30)}$ = 7.12, $p < 0.001$]. *Post hoc* analyses revealed that at 1 μM METH, only 1 μM GZ-793A significantly decreased [^{3}H]DA release and at 3 μM METH, GZ-793A (70 nM, 100 nM, and 1 μM) significantly decreased [^{3}H]DA release. At higher concentrations of METH, GZ-793A (70 nM – 1 μM) significantly decreased METH-evoked [^{3}H]DA release. Analysis of the log EC_{50} for METH-evoked [^{3}H]DA release revealed that GZ-793A (70 nM – 1 μM) increased the METH EC_{50} value [Table 1; $F_{(4,30)}$ = 26.6, $p < 0.0001$].

GZ-793A-induced inhibition of METH-evoked [^{3}H]DA release is not rate-dependent

To provide further evidence regarding the mechanism of GZ-793A-mediated inhibition of the effect of METH at synaptic vesicles, additional experiments determined if this inhibition was rate-dependent. The highest concentration of GZ-793A (1 μM), shown to selectively interact with High-affinity sites on VMAT2 (Fig. 2), was evaluated for inhibition of METH-evoked [^{3}H]DA release after 8- and 15-min incubation (Figure S1). Three-way repeated measures ANOVA revealed a main effect of METH [$F_{(4,32)}$ = 498, $p < 0.0001$] and GZ-793A [$F_{(1,8)}$ = 62.2, $p < 0.0001$], and a METH × GZ-793A interaction [$F_{(4,32)}$ = 39.2, $p < 0.0001$]; however, no main effect of time or interactions of METH × time, GZ-793A × time or METH × GZ-793A × time were observed. Increasing the incubation time from 8 to 15 min did not alter EC_{50} or E_{max} for METH-evoked [^{3}H]DA release.

Discussion

METH inhibits DA uptake at VMAT2 and evokes DA release from synaptic vesicles, increasing intracellular DA concentrations available for METH-induced reverse transport via DAT to ultimately increase extracellular DA concentrations (Sulzer *et al.* 2005). The lead compound emerging from our iterative drug discovery approach, GZ-793A, decreases METH-evoked DA release from superfused striatal slices and decreases METH self-administration, conditioned place preference, and cue-induced reinstatement (METH seeking behavior) in rats (Horton *et al.* 2011; Alvers *et al.* 2012; Beckmann *et al.* 2012). The cellular mechanisms underlying the GZ-793A-induced inhibition of effects of METH were investigated herein. The results show that GZ-793A potently released [^{3}H]DA from isolated striatal synaptic vesicles. In contrast to METH, GZ-793A-induced DA release was mediated by two sites on VMAT2, that is, a High-affinity, TBZ- and reserpine-sensitive site and a Low-affinity, TBZ- and reserpine-insensitive site. Moreover, GZ-793A inhibited METH-evoked [^{3}H]DA release from vesicles by interacting with High-affinity sites, that is, the intravesicular DA release site and the extravesicular DA uptake site on VMAT2. Thus, GZ-793A inhibits the effects of METH at VMAT2, which may underlie the previously reported GZ-793A-induced decrease in METH self-administration.

Previous research from our laboratories demonstrated that GZ-793A potently (K_i = 29 nM) and competitively inhibits [^{3}H]DA uptake at VMAT2 using isolated synaptic vesicle preparations. Interestingly, GZ-793A exhibited 285-fold higher affinity for the DA translocation site compared with the [^{3}H]DTBZ binding site (K_i = 8.29 μM), suggesting that inhibition of DA uptake is not via an interaction at the DTBZ site on VMAT2 (Horton *et al.* 2011). TBZ and reserpine have been shown to act at two different sites on VMAT2 (Yelin and Schuldiner 2002). Relative to the classical VMAT2 inhibitors, GZ-793A was found to be equipotent with TBZ and reserpine inhibiting DA uptake at VMAT2, but was 1-2-orders of magnitude less potent than TBZ and reserpine at the [^{3}H]DTBZ binding site (Partilla *et al.* 2006; Horton *et al.* 2011; Meyer *et al.* 2011; Nickell *et al.* 2011), consistent with these sites being different.

As GZ-793A exhibited a higher affinity for the DA translocation site compared with the [^{3}H]DTBZ binding site on VMAT2, GZ-793A inhibition of METH-evoked [^{3}H]DA release appeared to be due to inhibition of DA uptake at VMAT2. However, GZ-793A was significantly more potent (365-fold) inhibiting [^{3}H]DA uptake into vesicles than it was inhibiting METH-evoked DA release from striatal slices, warranting further evaluation of the cellular mechanism underlying the pharmacological effects of GZ-793A. Our working hypothesis was based on the idea that METH interacts with an extravesicular site on VMAT2 to inhibit DA uptake into the vesicle, and with an intravesicular site on VMAT2 to evoke DA release from the vesicle (Fig. 5). The current results show that GZ-793A also releases DA from the synaptic vesicle, presumably by interacting with intravesicular sites on VMAT2. Moreover, the biphasic concentration-response curve for GZ-793A to release [^{3}H]DA supports an interaction with two different intravesicular sites, a High-affinity site and a Low-affinity DA release site (Fig. 5), in contrast to METH. The current results also show that the intravesicular High-affinity DA release site for GZ-793A was both TBZ- and reserpine-sensitive. The Low-affinity site was insensitive to both TBZ and reserpine, suggesting that the Low-affinity site may represent a non-specific effect of GZ-793A on DA release, for example, disruption of the proton gradient responsible for retention of DA in the synaptic vesicle (Sulzer *et al.* 2005). The ability of TBZ and reserpine to inhibit GZ-793A-evoked DA release at the intravesicular High-affinity site appears to be via an allosteric interaction, as TBZ and reserpine act at different sites on VMAT2 (Pletscher 1977; Darchen *et al.* 1989; Yelin and Schuldiner 2002). Thus, TBZ and reserpine binding may conformationally change the VMAT2 protein resulting in inhibition of GZ-793A-evoked DA release.

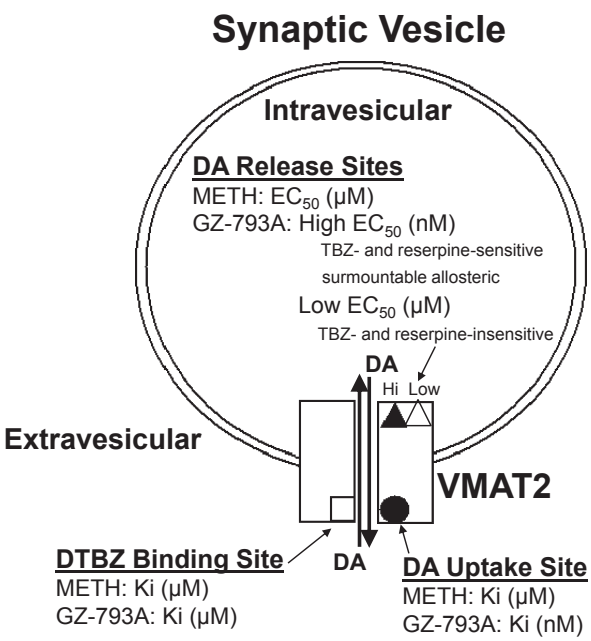

Fig. 5 GZ-793A interacts with multiple sites on vesicular monoamine transporter 2 (VMAT2). GZ-793A interacts with the extravesicular [^{3}H]DTBZ binding site (open square) with low affinity, the extravesicular [^{3}H]DA uptake site (closed circle) with high affinity, and intravesicular [^{3}H]DA release sites [tetrabenazine (TBZ)- and reserpine-sensitive High-affinity site, closed triangle; TBZ- and reserpine-insensitive Low-affinity site, open triangle]. Also illustrated is the proposed intravesicular site mediating GZ-793A-induced inhibition of METH-evoked DA release.

Concentration-response curves for both TBZ and reserpine to release DA were consistent with a one-site model of interaction (current results; Nickell *et al.* 2011), further indicating that GZ-793A acts differently than the classical VMAT2 compounds at the DA release site on VMAT2. Although GZ-793A, TBZ, and reserpine were equipotent at the extravesicular DA uptake site on VMAT2, the order of potency for DA release via the intravesicular site on VMAT2 was GZ-793A > TBZ > reserpine, suggesting that DA uptake and DA release are mediated by two different sites on VMAT2. Of note, GZ-793A interacts with the High affinity site mediating DA release across the same concentration range that it inhibits DA uptake by VMAT2 (High affinity DA release site, EC_{50} = 15 nM; DA uptake site, K_i = 29 nM; current results; Horton *et al.* 2011). Differential protein kinase C regulation of DA uptake and release sites on DAT (Gnegy 2003) provides precedence for alternate recognition sites on VMAT2 that mediate DA uptake into and DA release from the vesicle. Thus, GZ-793A interacts with at least three types of sites on VMAT2 (Fig. 5), i.e., intravesicular DA release sites, extravesicular DA uptake sites and extravesicular DTBZ binding sites.

The goal of the current work is to identify compounds which have efficacy decreasing the neurochemical effects of METH as potential pharmacotherapeutics to treat METH abuse. METH evokes DA release from synaptic vesicles, increasing the concentration of cytosolic DA available for reverse transport by DAT, leading to an increase in DA in the extracellular space (Sulzer *et al.* 2005). The current results demonstrate that low concentrations of the lead compound GZ-793A, which selectively interact with High-affinity intravesicular DA release sites and extravesicular DA uptake sites on VMAT2, also inhibit METH-evoked DA release from striatal synaptic vesicles. Results show that increasing concentrations of GZ-793A produced a rightward shift in the METH concentration response; however, the Schild regression revealed a slope different from unity, consistent with surmountable allosteric inhibition. Thus, at higher concentrations of METH, DA release is inhibited by GZ-793A and the maximal effect of these compounds is not additive. Surprisingly, at low concentrations METH inhibits the effect of GZ-793A to release DA.

Taken together and using classical pharmacological analyses, the current results show that GZ-793A interacts in a

surmountable allosteric manner. Precedence for surmountable allosteric inhibition has been provided by previous research on nicotinic and muscarinic acetylcholine receptor antagonists (Tucek and Proska 1995; Kukkonen *et al.* 2004; Wooters *et al.* 2011). Interpretations of concentration-response curves using Schild regression analysis are unambiguous with receptor binding data relative to functional data (Kenakin 1993). However, the distinction between ligand-gated ion channel receptors and transporters has become blurred with a greater understanding of these proteins (Galli *et al.* 1996; Sonders and Amara 1996; Sonders *et al.* 1997). In accordance with the characteristics of an allosteric mechanism of inhibition (Kenakin 2006), the shift to the right in the concentration response for METH-evoked DA release via VMAT2 should be diminished, as the allosteric site becomes saturated with increasing GZ-793A concentrations. Current results show a fivefold shift in EC_{50} as the GZ-793A concentration progressed from 7 to 70 nM, but only a one to twofold shift was apparent with GZ-793A concentrations ranging from 70 to 1000 nM, consistent with an allosteric mechanism. Further support for surmountable allosteric inhibition of METH-evoked DA release by GZ-793A is derived from the current observation that the inhibitory effect of GZ-793A was not rate dependent, as evidenced by no differences in the METH concentration-response curves in the presence of GZ-793A with increasing incubation time. Thus, GZ-793A inhibits METH by producing a conformational change in the VMAT2 protein, reducing the affinity of METH for the intravesicular DA release site, without altering efficacy of METH to release DA.

While GZ-793 shares pharmacological characteristics with classical VMAT2 inhibitors, TBZ and reserpine, there are also notable differences in their interaction with VMAT2. First, although GZ-793A, TBZ, and reserpine are equipotent and completely inhibit DA uptake at the extravesicular site on VMAT2 (Partilla *et al.* 2006; Horton *et al.* 2011; Nickell *et al.* 2011), inhibition produced by reserpine is irreversible (Rudnick 1990), whereas inhibition produced by TBZ and GZ-793A is not (Near 1986; data not shown). TBZ has been classified as a non-competitive inhibitor of the DA uptake site on VMAT2 (Scherman and Henry 1984). In agreement, previous results from our laboratory show that TBZ inhibits DA uptake through a surmountable allosteric mechanism (Nickell *et al.* 2011). GZ-793A inhibition of DA uptake at VMAT2 also has been shown to be surmountable (Horton *et al.* 2011), although a Schild analysis has not been carried out to determine if GZ-793A-induced inhibition of DA uptake is via an allosteric or orthosteric mechanism. Also, GZ-793A acts as a substrate for VMAT2 competing with DA for uptake, whereas TBZ does not act as a substrate, but acts as an uptake inhibitor. Second, these compounds differ in the order of potency for interaction at the extravesicular DTBZ binding site on VMAT2 (TBZ > reserpine > GZ-793A; Partilla *et al.* 2006; Horton *et al.* 2011), supporting the interpretation that GZ-793A acts differently than TBZ and reserpine. Third, with respect to the intravesicular DA release sites, GZ-793A exhibited a different pattern for the concentration-response curves compared to that for TBZ and reserpine. Specifically, the concentration-response curves for GZ-793A to evoke DA release from synaptic vesicles fit a two-site model of interaction, while those for TBZ and reserpine fit a one-site model. Furthermore, GZ-793A released DA with greater efficacy (E_{max} = 88%) than either TBZ or reserpine (E_{max} = 48.5 and 28.4%, respectively). Recent literature supports the existence of two pools of dopamine within the vesicle, a free pool and a pool associated with the ATP complex (Partilla *et al.* 2006). Compared with TBZ and reserpine, GZ-793A appears to have greater access to the ATP-associated DA pool within the vesicles. Moreover, GZ-793A inhibited METH-evoked DA release through interactions with the DA uptake site and the High affinity DA release site via a surmountable allosteric mechanism, while TBZ-induced inhibition of METH-evoked DA release is consistent with a competitive mechanism of action. Taken together, GZ-793A exhibits a unique pharmacological profile with respect to its interaction with VMAT2.

The action of GZ-793A to inhibit DA uptake into the synaptic vesicles and to increase release of DA from the vesicles presumably increases cytosolic DA concentrations leading to the expectation that GZ-793A may produce toxicity to DA neurons. However, GZ-793A interferes with METH-induced DA release from the vesicle by causing a decrease in the affinity of METH for VMAT2. Furthermore, using a superfused striatal slice assay, GZ-793A increases DOPAC extracellular concentrations, rather than increasing DA extracellular concentrations, in contrast to METH (Horton *et al.* 2011). Also, in contrast to METH, GZ-793A does not inhibit monoamine oxidase. Moreover, GZ-793A at a behaviorally relevant dose (15 mg/kg) which decreased METH self-administration, either administered acutely or repeatedly for 7 days does not deplete striatal DA tissue or vesicle content (unpublished data), also in contrast to METH. Moreover, GZ-793A does not exacerbate the depletion in DA content induced by a toxic regimen of METH (unpublished data). Thus, the ability of METH to inhibit monoamine oxidase likely contributes to the toxicity associated with METH, and is in contrast with GZ-793A, which does not inhibit monoamine oxidase and appears to lack toxicity to DA neurons.

In summary, GZ-793A likely interacts with distinct sites on VMAT2: (i) the extravesicular DTBZ binding site (low affinity), (ii) the extravesicular DA uptake site (high affinity) and (iii) intravesicular DA release sites (high and low affinity). GZ-793A inhibits METH-evoked DA release from synaptic vesicles by interacting with VMAT2 via a surmountable allosteric mechanism. There are a limited number of available compounds that interact with VMAT2. The addition of GZ-793A to our armamentarium has augmented our understanding of VMAT2 function and has identified a

specific pharmacological target to prevent METH's neurochemical action. GZ-793A represents a lead in the development of novel therapeutics for the treatment of METH abuse.

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Supporting information

Additional supporting information may be found in the online version of this article at the publisher's web-site:

Figure S1. GZ-793A-induced inhibition of METH-evoked DA release is not rate-dependent.

References

Alvers K. M., Beckmann J. S., Zheng G., Crooks P. A., Dwoskin L. P. and Bardo M. T. (2012) The effect of VMAT2 inhibitor GZ-793A on the reinstatement of methamphetamine-seeking in rats. *Psychopharmacology* **224**, 255–262.

Beckmann J. S., Denehy E. D., Zheng G., Crooks P. A., Dwoskin L. P. and Bardo M. T. (2012) The effect of a novel VMAT2 inhibitor, GZ-793A, on methamphetamine reward in rats. *Psychopharmacology* **220**, 395–403.

Damaj M. I., Patrick G. S., Creasy K. R. and Martin B. R. (1997) Pharmacology of lobeline, a nicotinic receptor ligand. *J. Pharmacol. Exp. Ther.* **282**, 410–419.

Darchen F., Scherman D. and Henry J. P. (1989) Reserpine binding to chromaffin granules suggests the existence of two conformations of the monoamine transporter. *Biochemistry* **28**, 113–122.

Di Chiara G. and Imperato A. (1988) Drugs abused by humans preferentially increase synaptic dopamine concentrations in the mesolimbic system of freely moving rats. *Proc. Natl Acad. Sci. USA* **85**, 5274–5278.

Dwoskin L. P. and Crooks P. A. (2002) A novel mechanism of action and potential use for lobeline as a treatment for psychostimulant abuse. *Biochem. Pharmacol.* **63**, 89–98.

Eisenhofer G., Kopin I. J. and Goldstein D. S. (2004) Leaky catecholamine stores: undue waste or a stress response coping mechanism? *Ann. N. Y. Acad. Sci.* **1018**, 224–230.

Fischer J. F. and Cho A. K. (1979) Chemical release of dopamine from striatal homogenates: evidence for an exchange diffusion model. *J. Pharmacol. Exp. Ther.* **208**, 203–209.

Galli A., Blakely R. D. and DeFelice L. J. (1996) Norepinephrine transporters have channel modes of conduction. *Proc. Natl Acad. Sci. USA* **6**, 8671–8676.

Gnegy M. E. (2003) The effect of phosphorylation on amphetamine-mediated outward transport. *Eur. J. Pharmacol.* **280**, 83–91.

Harrod S. B., Dwoskin L. P., Crooks P. A., Klebaur J. E. and Bardo M. T. (2001) Lobeline attenuates *d*-methamphetamine self-administration in rats. *J. Pharmacol. Exp. Ther.* **280**, 1432–1444.

Harrod S. B., Dwoskin L. P., Green T. A., Gehrke B. J. and Bardo M. T. (2003) Lobeline does not serve as a reinforcer in rats. *Psychopharmacology* **165**, 397–404.

Horton D. B., Siripurapu K. B., Zheng G., Crooks P. A. and Dwoskin L. P. (2011) Novel N-1,2-dihydroxypropyl analogs of lobelane inhibit vesicular monoamine transporter-2 function and methamphetamine-evoked dopamine release. *J. Pharmacol. Exp. Ther.* **339**, 1–12.

Jones R. (2007) Double-blind, placebo-controlled, cross-over assessment of intravenous methamphetamine and sublingual lobeline interactions. NCT00439504. Clinical Trials.gov.

Kenakin T. P. (1993) *Pharmacologic Analysis of Drug-Receptor Interaction*, 2nd edn. Raven Press, New York.

Kenakin T. P. (2006) *A Pharmacology Primer, Second Edition: Theory, Application, and Methods*, 2nd edn. Academic Press, San Diego.

Kukkonen A., Perakyla M., Akerman K. E. O. and Nasman J. (2004) Muscarinic toxin 7 selectivity is dictated by extracellular receptor loops. *J. Biol. Chem.* **279**, 50923–50929.

Meyer A. C., Horton D. B., Neugebauer N. M., Wooters T. E., Nickell J. R., Dwoskin L. P. and Bardo M. T. (2011) Tetrabenazine inhibition of monoamine uptake and methamphetamine behavioral effects: locomotor activity, drug discrimination and self-administration. *Neuropharmacology* **61**, 849–856.

Miller D. K., Crooks P. A., Zheng G., Grinevich V. P., Norrholm S. D. and Dwoskin L. P. (2004) Lobeline analogs with enhanced affinity and selectivity for plasmalemma and vesicular monoamine transporters. *J. Pharmacol. Exp. Ther.* **310**, 1035–1045.

Near J. A. (1986) [^{3}H]Dihydrotetrabenazine binding to bovine striatal synaptic vesicles. *Mol. Pharmacol.* **30**, 252–257.

Neugebauer N. M., Harrod S. B., Stairs D. J., Crooks P. A., Dwoskin L. P. and Bardo M. T. (2007) Lobelane decreases methamphetamine self-administration in rats. *Eur. J. Pharmacol.* **571**, 33–38.

Nickell J. R., Krishnamurthy S., Norrholm S., Deaciuc A. G., Siripurapu K. B., Zheng G., Crooks P. A. and Dwoskin L. P. (2010) Lobelane inhibits methamphetamine-evoked dopamine release via inhibition of the vesicular monoamine transporter-2. *J. Pharmacol. Exp. Ther.* **332**, 612–621.

Nickell J. R., Zheng G., Deaciuc A. G., Crooks P. A. and Dwoskin L. P. (2011) Phenyl ring-substituted lobelane analogs: inhibition of [^{3}H] dopamine uptake at the vesicular monoamine transporter-2. *J. Pharmacol. Exp. Ther.* **336**, 724–733.

Partilla J. S., Dempsey A. G., Nagpal A. S., Blough B. E., Baumann M. H. and Rothman R. B. (2006) Interaction of amphetamines and related compounds at the vesicular monoamine transporter. *J. Pharmacol. Exp. Ther.* **319**, 237–246.

Peter D., Liu Y., Brecha N. and Edwards R. H. (1995) The transport of neurotransmitters into synaptic vesicles. *Prog. Brain Res.* **105**, 273–281.

Pifl C., Drobny H., Reither H., Hornykiewicz O. and Singer E. A. (1995) Mechanism of the dopamine-releasing actions of amphetamine and cocaine: plasmalemmal dopamine transporter versus vesicular monoamine transporter. *Mol. Pharmacol.* **47**, 368–373.

Pletscher A. (1977) Effect of neuroleptics and other drugs on monoamine uptake by membranes of adrenal chromaffin granules. *Br. J. Pharmacol.* **59**, 419–424.

Rudnick G. (1990) Energetics of reserpine binding and occlusion by the chromaffin granule biogenic amine transporter. *Biochemistry* **29**, 603–608.

Scherman D. and Henry J. P. (1984) Reserpine binding to bovine chromaffin granule membranes. Characterization and comparison with dihydrotetrabenazine binding. *Mol. Pharmacol.* **25**, 113–122.

Schuldiner S., Steiner-Mordoch S., Yelin R., Wall S. C. and Rudnick G. (1993) Amphetamine derivatives interact with both plasma membrane and secretory vesicle biogenic amine transporters. *Mol. Pharmacol.* **44**, 1227–1231.

Sonders M. S. and Amara S. G. (1996) Channels in transporters. *Curr. Opin. Neurobiol.* **6**, 294–302.

Sonders M. S., Zhu S. J., Zahniser N. R., Kavanaugh M. P. and Amara S. G. (1997) Multiple ionic conductances of the human dopamine

transporter: the actions of dopamine and psychostimulants. *J. Neurosci.* **17**, 960–974.

Substance Abuse and Mental Health Services Administration. (2011) *Results from the 2010 National Survey on Drug Use and Health: Volume I. Summary of National Findings* (Office of Applied Studies, NSDUH Series H-41, HHS Publication No. SMA 11-4856 Findings). Rockville, MD.

Sulzer D. and Rayport S. (1990) Amphetamine and other psychostimulants reduce pH gradients in midbrain dopaminergic neurons and chromaffin granules: a mechanism of action. *Neuron* **5**, 797–808.

Sulzer D., Sonders M. S., Poulsen N. W. and Galli A. (2005) Mechanisms of neurotransmitter release by amphetamines: a review. *Prog. Neurobiol.* **75**, 406–433.

Teng L. H., Crooks P. A., Sonsalla P. K. and Dwoskin L. P. (1997) Lobeline and nicotine evoke [^{3}H]overflow from rat striatal slices preloaded with [^{3}H]dopamine: differential inhibition of synaptosomal and vesicular [^{3}H]dopamine uptake. *J. Pharmacol. Exp. Ther.* **280**, 1432–1444.

Teng L. H., Crooks P. A. and Dwoskin L. P. (1998) Lobeline displaces [^{3}H]dihydrotetrabenazine binding and releases [^{3}H]dopamine from rat striatal synaptic vesicles: comparison with d-amphetamine. *J. Neurochem.* **71**, 258–265.

Tucek S. and Proska J. (1995) Allosteric modulation of muscarinic acetylcholine receptors. *Trends Pharmacol. Sci.* **16**, 205–212.

Wise R. A. and Bozarth M. A. (1987) A psychomotor stimulant theory of addiction. *Psychol. Rev.* **94**, 469–492.

Wooters T. E., Smith A. M., Pivavarchyk M., Siripurapu K. B., McIntosh J. M., Zhang Z., Crooks P. A., Bardo M. T. and Dwoskin L. P. (2011) bPiDI: a novel selective α6β2* nicotinic receptor antagonist and preclinical candidate treatment for nicotine abuse. *Br. J. Pharmacol.* **163**, 346–357.

Yelin R. and Schuldiner S. (2002) Vesicular neurotransmitter transporters pharmacology, biochemistry and molecular analysis, in *Contemporary Neuroscience: Neurotransmitter Transporters: Structure, Function, and Regulation* (Reith M. E. A., ed.), pp. 313–354. Humana Press, Totowa, NJ.